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Original Article

Tryptophan Negatively Regulates IgE-mediated Mast Cell Activation

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Abstract : Mast cells are major immune cells in allergy to secrete allergic mediators by a degranulation process and make and secrete inflammatory lipids and cytokines in response to antigen stimulation. An amino acid tryptophan regulates immune functions. Tryptophan ameliorates inflammatory colitis in which mast cells are engaged. However, its effects on mast cells remain to be solved.

We investigated the effect of tryptophan on IgE-mediated allergic responses in the mast cells and mice. IgE-mediated passive cutaneous anaphylaxis (PCA) in mice were examined. Also IgE-mediated mast cell activation responses such as degranulation of stored granules and secretion of inflammatory lipid LTB_4 and cytokines (TNF-α and IL-4) were measured.

Intraperitoneal administration of tryptophan suppressed PCA in mice. Also, in the cellular level tryptophan inhibited IgE-mediated mast cell activation such as IgE-mediated degranulation and the production of LTB_4. Also, it inhibited production of inflammatory cytokines TNF-α and IL-4.

In summary, tryptophan suppressed IgE-mediated allergic activation in vivo and in vitro. Tryptophan supplementation is beneficial for IgE-mediated allergy.

Keywords : Passive cutaneous anaphylaxis, Mast cell, Degranulation, IgE, Tryptophan

Introduction

Mast cells are key effector cells associated with IgE-mediated allergic diseases [1,2]. Mast cells express FcεRIs, the high affinity receptor for IgE [3]. FcεRIs that are occupied with IgEs are cross-linked by the antigen treatment. These interaction initiates signaling events resulting in allergic reaction. Stimulated cells degranulate secretary granules that store preformed inflammatory mediators such as histamine, TNF-α and proteases. Also, they synthesize and secrete inflammatory lipids and cytokines such as LTB_4, TNF-α and IL-4. In addition to its allergic role, mast cells act as effector and immunregulatory cells in tumor biology, host defense, cardiovascular disease, limitation of inflammation and tissue pathology [4].

Tryptophan is an essential amino acid and its depletion or metabolites through dioxygenation reaction regulates immune responses [5]. Tryptophan suppresses dextran

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sodium sulfate-induced colitis in a porcine model [6] in which mast cells are involved [7]. Tryptophan can be metabolized for the synthesis of NAD+ which is a substrate of protein deacetylation reaction consequently regulates cellular functions such as senescence, survival, DNA repair, metabolism and proliferation [8]. NAD+ is convertible to nicotinamide which is able to inhibit anaphylactic mast cell degranulation in mice by the treatment of compound 48/80, a mast cell activator [9]. However, tryptophan effects on mast cell activation have not been studied.

In this study we investigated the effect of tryptophan on IgE-mediated allergic responses in mice and mast cells. We show that tryptophan suppresses IgE-mediated passive cutaneous anaphylaxis (PCA) in mice and allergic reactions in mast cells such as IgE-mediated degranulation and secretion of an inflammatory lipid and cytokines.

Materials and Methods

1. Materials

The following materials were purchased from the indicated commercial sources: MTT formazan, ketotifen, tryptophan, dinitrophenol (DNP)-specific monoclonal IgE, DNP-HSA and Evans blue dye from Sigma-Aldrich; fetal bovine serum from Gibco/Life Technology; Eagle's minimal essential medium (EMEM) and trypsin from Lonza; recombinant IL-3 from Peprotech; anti-phospho-ERK (Thr202/Tyr204) from Cell Signaling Technology; ELISA sets for mouse TNF-α and IL-4 cytokine from BD Biosciences; LTB_4 ELISA kit from Enzo Life sciences; a rat basophilic leukemia cell line (RBL-2H3) from American Type Culture Collection.

2. Induction of passive cutaneous anaphylaxis in mice

The BALB/c mice were purchased from DBL Korea and kept under specific pathogen-free conditions and maintained according to the guidelines of the Institutional Animal Care and Use Committee of Chungnam National University. PCA was performed as previously described with some modifications [10]. Briefly, the mice were anaesthetized and injected dermally with DNP-specific IgE (0.5 μg each mouse) into the ear. After 24 h, DNP-HSA antigen (250 μg) in 200 μL PBS containing 1% Evans blue was injected intravenously. Tryptophan or Ketotifen (a commercial anti-allergic drug) was administered via peritoneal injection 1 h before antigen treatment. The mice were sacrificed 1 h after antigen challenge, followed by the removal of ears for measurement of the amount of dye extravasated. The dye was extracted overnight from the ears in 500 μL of formamide at 63ºC. The absorbance was measured in a microplate reader at 620 nm.

3. Preparation and culture of bone marrow-derived mast cells and RBL-2H3 cells

For the preparation of bone marrow-derived mast cells (BMMCs), femurs of 6 weeks old BALB/c mouse were taken and bone marrow was flushed with PBS and collected. The cells were suspended and cultured in RPMI 1640 medium containing 10% heat inactivated FBS, 1% penicillin and streptomycin, 2 mM glutamine, 50 μM 2-mercaptoethanol, 25 mM HEPES [pH 7.4] and supplemented with 10 ng/mL IL-3 in 5% CO_2 incubator at 37ºC. The non-adherent cells were transferred to the fresh medium and cultured twice a week. By 4∼5 weeks in culture, the cells were used for further experiments after verifying that cells have granules stained with toluidine blue. RBL-2H3 cells were cultured in complete media (EMEM supplemented with 15% (v/v) FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin) at 37ºC in 5% CO_2 incubator.

4. Sensitization and stimulation of BMMCs and RBL-2H3 mast cells

RBL-2H3 cells and BMMCs were sensitized with 100 ng/mL DNP-specific IgE overnight in a complete growth medium. The cells were stimulated with 100 ng/mL DNP-HAS antigen after washing and preincubation for 30 min with Tyrode's buffer (10 mM HEPES [pH 7.4], 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 5.6 mM glucose) to measure degranulation, LTB_4 and activation of ERK. To measure cytokine mRNAs, cytokines and cell viabilities, a complete growth medium was used to wash and preincubate the cells.

5. Measurement of degranulation of mast cells

The activity of granule marker enzyme β-hexosaminidase in the medium and inside the cells after antigen stimulation was determined by a colorimetric assay [10] with some modification. Briefly, cells were transferred to 24-well (1×10^5 cells per 0.4 mL per well) cluster plates and

sensitized overnight. The cultures were washed twice and replaced with Tyrode's buffer (0.2 mL per well) for RBL-2H3 cells and BMMCs. The cultures were incubated for 1 h with or without tryptophan before stimulation with antigen for 20 min. Production of *p*-nitrophenol from 5 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide was measured by a colorimetric assay. The values were expressed as percentages of secreted β-hexosaminidase activity per total activities (intracellular plus secreted activities). MTT assay to measure cytotoxic effect of tryptophan was performed according to the protocol of a MTT manufacturer, Sigma-Aldrich.

6. Measurement of LTB_4 release by ELISA

RBL-2H3 cells were cultured in 24-well (1×10^5 cells per 0.4 mL per well) cluster plates, sensitized and stimulated for 30 min as described in the 4 section. The released LTB_4 into the medium after antigen stimulation for 30 min was measured by ELISA assay according to the protocol supplied by Enzo Life Sciences.

7. ELISA of TNF-α and IL-4

BMMCs were transferred to 6-well (1×10^5 cells per 2 mL per well) cluster plates, sensitized and stimulated as described in section 4. The cells were stimulated with antigen for 6 h after pre-incubation with tryptophan for 30 min. TNF-α and IL-4 in the supernatants were measured using the ELISA kit according to the manufacturer's protocol.

8. Immunoblotting analysis

RBL-2H3 cells (2×10^5 cells per 2 mL per well) were cultured in 6-well cluster plate, sensitized and stimulated as described in section 4. Then cells were washed twice with ice-cold PBS, and then lysed in ice-cold lysis buffer for 30 min on ice [11]. Lysates were micro-centrifuged at 12,000 rpm at 4°C for 20 min. Equal amount of proteins was mixed with 4× sample buffer, boiled for 5 min, subjected to electrophoresis in the SDS-PAGE gel and then transferred to the PVDF membrane. The membrane was blocked in 5% skim milk in Tris-buffered saline-Tween 20 (TBS-T) for 1 h and then incubated overnight with primary antibodies at 4°C. After washing the membrane with TBS-T, immune-reactive proteins were detected with horseradish peroxidase-conjugated secondary antibody using chemiluminescence kit.

9. Statistical analysis

Statistical analysis was performed with data from three independent experiments. Data are presented as means ± SEM. One way ANOVA followed by Dunnett's test was used to calculate the significant differences between treatment groups in in vivo experiments. In case of in vitro experiments, unpaired Student's *t*-test was performed. $P \leq 0.05$ was considered to indicate significance.

Results

1. Tryptophan suppressed IgE-mediated passive cutaneous anaphylaxis in mice

We examined the anti-allergic activity of tryptophan in IgE-mediated PCA in the mouse ears. PCA is a well-established IgE-mediated type I immediate hypersensitivity allergy model that mast cells act as main cells [12]. Allergic reaction makes blood vessels leaky to extravasate. Intravenous administration of the specific antigen elicits PCA in mice ears where the antigen specific-IgE was subcutaneously injected 1 day before. Evans blue dye from the vessels in the IgE-sensitized ears leaked after stimulation with antigen solution. Peritoneal administration of tryptophan 1 h before the antigen challenge attenuated the PCA reaction in a dose-dependent manner (Fig. 1). Also, commercial mast cell stabilizer Ketotifen suppressed PCA. These results suggest that tryptophan inhibit IgE-mediated PCA in mice like an anti-allergic medicine Ketotifen does.

2. Tryptophan and its metabolites inhibited IgE-mediated degranulation of mast cells

Mast cells are the main allergic cells under the skin [13]. Effects of tryptophan on IgE-mediated mast cell activation were examined in RBL-2H3 cell line. Mast cells secreted a secretary granule enzyme, β-hexosaminidase, through degranulation process after antigen treatment (Fig. 2A). Administration of tryptophan suppressed the degranulation in a dose-dependent manner (Fig. 2A) without any cytotoxic effect (Fig. 2C). These data suggest that tryptophan inhibit IgE-mediated mast cell degranulation.

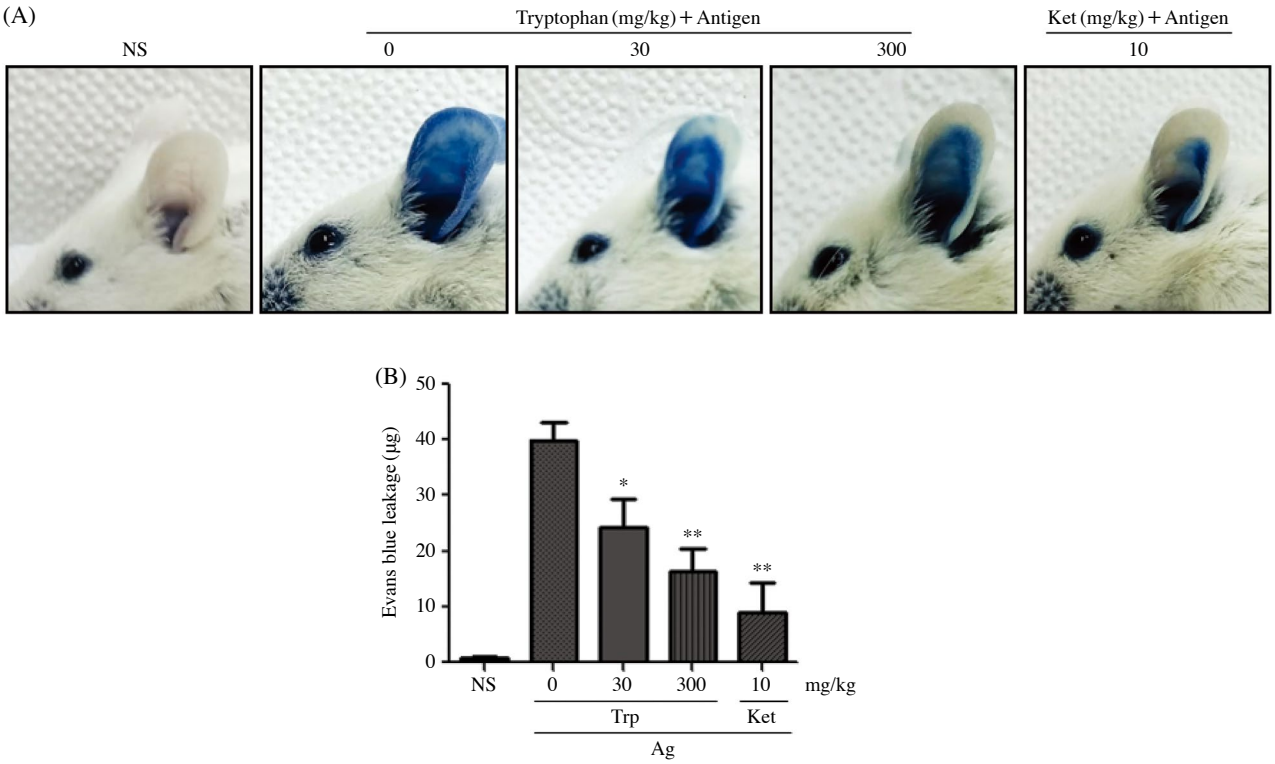

Fig. 1. Tryptophan suppresses IgE-mediated passive cutaneous anaphylaxis (PCA) in mice. (A) The leak of Evans blue dye in IgE-sensitized ears by an anaphylaxis reaction was suppressed by the intraperitoneal treatment of tryptophan 1 h before the antigen challenge. Each picture shown is a representative of 3 independent experiments. (B) The quantities of leaked Evans blue dye from the ears were measured and graphed. The means ± SEMs of values from 3 independent PCA experiments, each with 10 mice, are shown. Significant difference against control group is indicated as *($P < 0.05$) and **($P < 0.01$). NS is no stimulation. Ket is ketotifen fumarate (10 mg/kg). Ag is antigen.

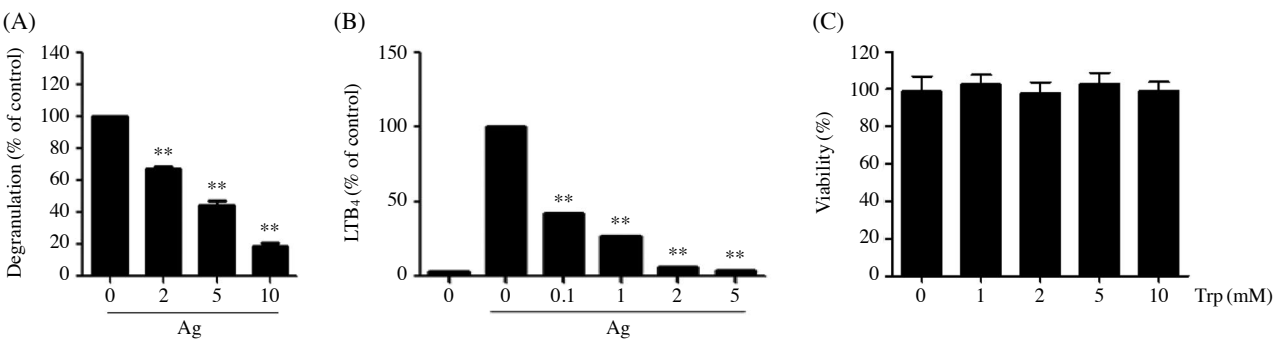

Fig. 2. Tryptophan inhibits IgE-mediated degranulation and secretion of LTB_4 in RBL-2H3 mast cells. The IgE-sensitized mast cells were stimulated with antigen to measure the secretion of allergic mediators to the culture medium. Degranulation and LTB_4 secretion were measured after antigen stimulation for 30 min. Tryptophan treatment for 1 h inhibited the degranulation in a dose dependent manner (A) and suppressed secretion of LTB_4 (B) without any cytotoxic effect to cells (C). The data are mean ± SEM from 3 independent experiments. Significant difference against control group is indicated as *($P < 0.05$) and **($P < 0.01$).

3. Tryptophan inhibited LTB_4 secretion in antigen-stimulated mast cells

Activated mast cells synthesize and release inflammatory eicosanoids such as prostaglandins and leukotrienes that mediate allergic responses [14]. Cytosolic phosphlipase A2 (cPLA2) hydrolyzes phospholipds to produce arachidonic acid that is metabolized to the inflammatory

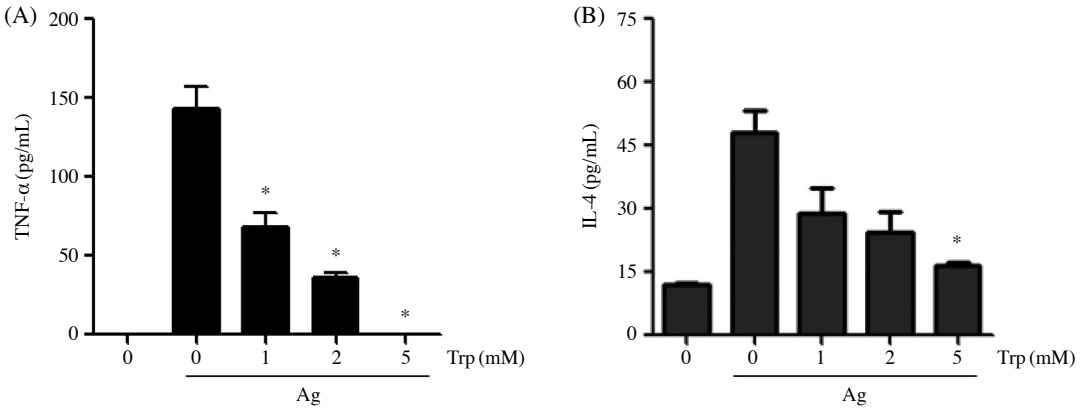

Fig. 3. Tryptophan suppresses the expression and secretion of TNF-α and IL-4 from bone marrow derived mast cells (BMMCs). IgE-sensitized BMMCs were stimulated for 6 h with the antigen. Tryptophan suppressed the secretion of TNF-α (A) and IL-4 (B) to the culture medium in a dose dependent manner. Significant difference against a control group is indicated as $*P<0.05$.

eicosanoids, such as prostaglandin and leukotriene [14]. Leukotriene B4 (LTB_4) is the most potent known chemoattractants, acting primarily on neutrophils, eosinophils, T cells, and mast cells [15]. We examined whether tryptophan inhibits secretion of LTB_4. Tryptophan suppressed secretion of LTB_4 in a dose-dependent manner in antigen-stimulated RBL-2H3 cells. The IC_{50} value was approximately 0.91 ± 0.27 mM (Fig. 2B). Nearly complete suppression of LTB_4 secretion was achieved with 2 mM tryptophan.

4. Tryptophan inhibited inflammatory cytokine production in bone marrow derived mast cells

Activated mast cells are the source of various cytokines such as TNF-α and IL-4 which are involved in allergic inflammation [1]. We examined whether tryptophan suppresses cytokine production of mast cells. For cytokine production experiment, we used bone marrow-derived mast cells (BMMC) because this primary cells are good for IgE-mediated cytokine production assay. BMMCs secreted inflammatory cytokines TNF-α and IL-4 in response to antigen stimulation. Tryptophan suppressed the secretion of the cytokines in a dose-dependent manner (Fig. 3B).

5. Tryptophan inhibited IgE-mediated intracellular signaling in antigen-stimulated mast cells

IgE-mediated mast cell activation is initiated by cross-linking of high affinity receptors for IgE (FcεRIs) on mast cells [16]. The cross-linking of the receptors activates Src-family tyrosine kinases and subsequently Syk that is critical for downstream signaling events resulting in activation of MAPKs such as JNK, p38 and ERK. We examined the ERK activation because it is required for the production of inflammatory lipids and cytokines. Antigen stimulation induced ERK phosphorylation. Pretreatment of tryptophan attenuated ERK phosphorylation in a dose-dependent manner (Fig. 4).

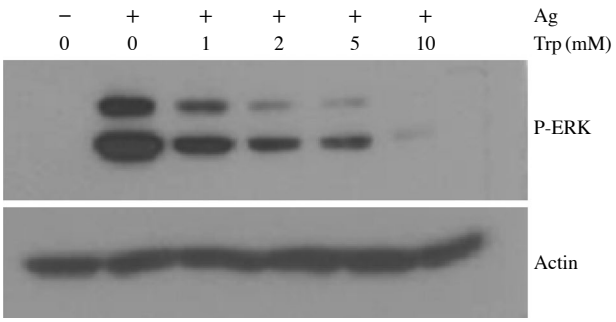

Fig. 4. Tryptophan suppresses activating phosphorylations of an MAP kinase ERK. The IgE-sensitized RBL-2H3 cells were stimulated with antigen for 20 min. Cell lysates were used for immunoblots with the antibody specific to phosphorylated ERK. Tryptophan treatment attenuated ERK phosphorylation in a dose dependent manner. Immunoblots shown are the representative of 3 independent experiments.

Discussion

In this study we observed that tryptophan inhibits IgE-

mediated degranulation and secretion of inflammatory lipid LTB_4 and cytokines TNF-α and IL-4 and suppresses IgE-mediated PCA. Because these molecules are major mediators of IgE-mediated allergic responses [13], our results show that tryptophan inhibits mast cell-mediated allergy.

Peritoneal treatment of tryptophan inhibited IgE-mediated PCA in mice in a dose dependent manner (Fig. 1). Because mast cells are major mediator cells in PCA, we examined the tryptophan effect on IgE-mediated activation of mast cells in cultures. Pretreatment of tryptophan 1 h before the antigen stimulation is required for the maximal inhibition of IgE-mediated degrnulation and LTB_4 (Fig. 2). During tyrptophan treatment for 1 h, tryptophan may be partly metabolized to kynurenine which is a tryptophan metabolite formed during tryptophan degradation process to make nicotinamide and NAD+. The anti-allergic effects of tryptophan in this study may come from actions of tryptophan metabolites formed in mast cells. However, there is a report that a tryptophan metabolite, kynurenine, is marginally pro-allergic [17]. The report proposed that kynurenine enhanced allergic responses in mast cells and PCA through its genomic action as an agonist of an aryl hydrocarbon receptor, a transcription factor based on observations with knock-out mice and mast cells in an aryl hydrocarbon receptor gene. Knock-out mice are a useful tool but there is probability that they might have developmental defect resulting in the animal behaving differently from wild type mice. In this study, tryptophan showed anti-allergic effects. We don't know yet that anti-allergic effect of tryptophan come from tryptophan itself or its metabolites.

We observed that tryptophan inhibits IgE-mediated ERK activation (Fig. 4). cPLA2 is critical for allergic responses [18]. Phosphorylation of cPLA2 by ERK is important to its catalytic activity. Among the inhibitory effect of tryptophan on degranulation (Fig. 2A), secretion of LTB_4 (Fig. 2B), TNF-α (Fig. 3A) and IL-4 (Fig. 3B) in activated mast cells, LTB_4 secretion is mostly susceptible to tryptophan treatment. IgE-mediated FcεRI signaling is initiated from activation of tyrosine kinases and inactivation of them blocks every signaling events leading to degranulation and secretion of inflammatory cytokines and eicosanoids [3]. Therefore we speculated that tryptophan targets in mast cells are multiple.

In summary, tryptophan suppressed PCA potently in mice also IgE-mediated allergic responses of mast cells such as degranulation and secretion of LTB_4, TNF-α and IL-4. From our present data, we suggest that tryptophan suppresses IgE-mediated allergic reaction.

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트립토판은 IgE-매개 비만세포 활성화를 억제한다

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간추림 : 비만세포는 알레르기 반응을 일으키는 주된 세포로서 항원 자극에 반응하여 알레르기 유발 물질인 히스타민, 단백질 분해효소, 염증성 지질 물질 및 사이토카인 등을 분비한다. 아미노산인 트립토판은 염증반응을 조절한다. 트립토판 투여는 비만세포가 관여하는 염증성 장염 증상을 완화시킨다. 그러나 트립토판이 비만세포의 알레르기 반응에 미치는 영향에 대한 연구는 없다.

본 저자들은 트립토판이 IgE 매개 알레르기 반응에 미치는 영향을 비만세포 수준에서 그리고 실험동물 생쥐에서 연구하였다. IgE-매개 수동 피부 아나필락시스를 생쥐에서 연구하였다. 또한 IgE-매개 비만세포 활성화 반응 즉, 탈과립 반응, 염증성 지질인 LTB_4와 사토카인(TNF-α와 IL-4) 등의 분비를 측정하였다.

트립토판을 생쥐에 복강 주사하면 IgE 매개 수동 피부 아나필락시스를 억제하였다. 또한 비만세포 수준에서도 트립토판은 IgE 매개 알레르기 반응들, 즉 탈과립 반응과 염증성 지질인 LTB4 및 사이토카인인 TNF-α와 IL4의 분비를 억제하였다.

이러한 결과로부터 트립토판은 IgE 매개 알레르기 반응을 세포 수준 및 실험동물 수준에서 억제함을 알 수 있었다.

찾아보기 낱말 : 수동 피부 아나필락시스, 비만세포, 탈과립, IgE, 트립토판

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